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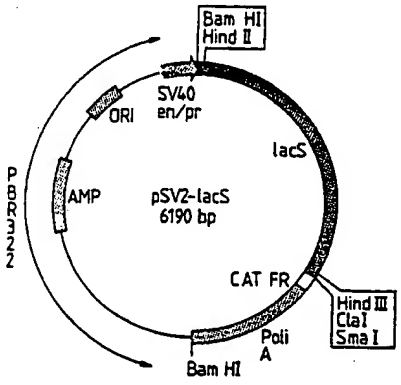
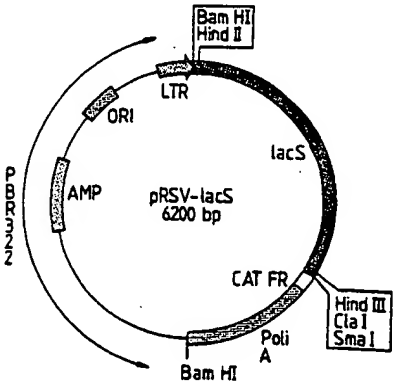
(57) Abstract

A recombinant protein or peptide expression system comprises, as reporter gene, a gene coding for an enzyme or other protein which is stable under conditions, e.g. of temperature, in which at least some and preferably all of the other protein or peptide products of the system are inactivated.





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NEW REPORTER GENES

This invention relates to gene expression in eukaryotic organisms, and more particularly to novel reporter genes for use in such systems and to their use.

In eukaryotic organisms, gene expression is mainly controlled by the transcription mechanism. RNA polymerase II catalyzes the synthesis of mRNA from the structural DNA genes through the formation of a complex with specific target DNA sequences (promoters) and protein factors which are responsible for the correct initiation of the transcription. Promoters for RNA polymerase II contain a variety of short cis-acting DNA sequences which are specifically recognised by transacting protein factors. These modular DNA sequences are located mainly upstream of the TATA box (when present) but can be placed in both orientations and at very variable distances from the transcription start site. The proximal region to this site is responsible for correct initiation of transcription, while the regions further upstream determine the transcriptional efficiency of the overall promoter. Some of these promoter sequences are contained in several genes and are recognised by widely present protein factors. Other promoter sequences, on the other hand, are characteristic of specific groups of genes and interact with specific transcription factors: they are called "responsive elements" (RE).

The ability to determine the transcripti nal activity of cloned genomic sequences after their introduction into appropriate cells has substantially enriched understanding of how mammalian gene transcription is regulated. Such studies have been greatly simplified by the use of vectors containing reporter genes.

Reporter genes code for proteins which possess a unique enzymatic activity or are otherwise easily distinguishable from the mixture of intra- and extra-cellular proteins produced by the expression system under study. The basic strategy for analysing the efficiency of gene transcription using reporter genes is as follows. To test the efficacy of the promoter elements of the expression system, the DNA under test is ligated upstream of the coding region of the reporter gene so as to produce a chimeric gene in which the putative regulatory elements control the expression of the reporter gene. Similarly, to test for functional enhancer elements, which by definition can increase transcription when placed upstream or downstream and in either orientation relative to the promoter, the test DNA can be ligated upstream or downstream of a reporter gene which already is associated with a promoter. The fused genes so obtained are subsequently introduced, by any of a variety of techniques, into cultured cells or into animals, e.g. to produce transgenic mice. The transcriptional capability of the DNA

under t st is then estimated quantitatively from the in vitro activity of the reporter gene product in the culture medium or in cellular or tissue-derived extracts, or is determined qualitatively or by histochemical techniques of intact cells.

The basic technique may be varied to take account of the particular system being studied. Although the activity or quantity of the reporter gene product is an indirect measurement of the transcriptional properties of the DNA under test, generally the reporter gene activity is directly proportional to the transcriptional activity.

The vectors containing reporter genes used up till now have certain properties in common. They often have a backbone derived from pBR322 or pUC, which allow for propagation and selection in Escherichia coli. In addition, such vectors generally possess a eucaryotic poly(A) addition signal and an intron, either intrinsic to the reporter gene or derived from a heterologous source. The presence of an intron is necessary for efficient production of some mature cytoplasmic mRNAs. Unique cloning sites, located upstream or downstream of the reporter gene, are designed for easy insertion of promoter and RE sequences to be tested.

Up till now three main types of reporter genes have generally been used:

(a) Chloramphenicol Acetyltransferase. One widely used

reporter gene codes f r chl ramph nicol ac tyltransferas (CAT) in E. coli(1). The CAT gene is not endogenous to mammalian cells and the enzyme which it produces can be easily assayed and its activity detected at low levels. CAT catalyses the transfer of acetyl groups from acetyl coenzyme A (acetyl-CoA) to chloramphenicol. Mono- and diacetyl forms of chloramphenicol can be produced. Since acetylated and unacetylated forms of chloramphenicol possess different solubilities in organic solvents, they can be separated by silica gel thin-layer chromatography (TLC) or by differential extraction in organic solvents. Moreover, the acetylated forms can be radiolabelled by incubating (^{14}C)chloramphenicol with acetyl-CoA, or, less expensively, incubating ordinary chloramphenicol with (^{3}H)acetyl-CoA in the test reaction. The amount of acetylation can be measured by densitometry or by liquid scintillation counting of the appropriate region of the silica gel after TLC or the organic phase after differential extraction.

(b) Luciferase. The DNA sequence coding firefly (Photinus pyralis) luciferase can be used in a very versatile reporter gene system (2,3). Luciferase catalyses the reaction:

Luciferin + ATP + O_2 → oxyluciferin + AMP + PP_i + CO_2 + light

Under appropriate conditions, both the maximum light intensity and the total light output ov r a period of time

are proportional to the concentration of luciferase in the reaction system. The light emission can be measured by a luminometer or by liquid scintillation.

(c) Beta-Galactosidase. β-galactosidase from E. coli is a tetramer with a subunit size of 1023 amino acids. It catalyses the hydrolysis of various β-galactosides, including lactose. Measurement of β-galactosidase activity provides a useful internal control for measuring the variability in reporter enzyme activity caused by differences in transfection efficiency or cell extract preparation(4). A simple photometric assay method may be used to measure hydrolysis of the substrate ONPG (o-nitrophenyl βD-galactopyranoside) by β-galactosidase in a cell-free system. The recent discovery of substrates for β-galactosidase which generate fluorescent products has significantly increased the sensitivity of this assay.

Nevertheless, there are serious disadvantages in all the known reporter gene systems:

(1) Although the luciferase system is very sensitive, the luminometer required to measure the enzyme activity is expensive and not very widely available;

(2) mRNA from the reporter gene and consequently enzymatic activity, can be generated by read-through transcription or by spurious initiation of the transcription from within the plasmid-associated procaryotic sequences. It has been demonstrated that many

plasmid constructions containing CAT as reporter gene may drive transcription initiation at several unspecific sites, leading to spuriously increased CAT activity. In addition, the assay method is quite complicated and not easy to reproduce. Use of radio labelled compounds requires special laboratory licences.

(3) Many cells or tissues (e.g. epithelial cells) endogenously produce β-galactosidase. Precise measurement of exogenous β-galactosidase activity can therefore be difficult.

The present invention provides a novel class of reporter genes, the use of which avoids at least some of the difficulties already mentioned. A recombinant protein or peptide expression system in accordance with the present invention comprises, as a reporter gene, a gene coding for an enzyme or other protein which is stable under conditions, e.g. of temperature, in which at least some, and preferably all, of the other protein or peptide products of the said system are denatured or otherwise inactivated. The expression system includes, in addition to the reporter gene, a promoter and/or other DNA sequences which do not encode a protein or peptide product but assist in the expression of the reporter gene. The use of genes coding for highly thermostable enzymes represents a novel and advantageous strategy for the study of the efficiency of expression of eucaryotic cis-acting genomic sequences.

Using reporter genes which give rise to products which are intrinsically stable to temperature and protein denaturants allows the quick and efficient removal, by suitable selective denaturation, of any interfering proteins which are endogenously expressed by the host cells.

Use of reporter genes encoding for enzymes or other proteins which are stable under extreme conditions offers the following advantages:

(a) The new systems may be used in cell systems which endogenously express high levels of an interfering enzyme (e.g. the endogenous β-gal activity of epithelial cells) which is not stable under extreme conditions.

(b) Since background enzymatic activity can be removed, the accuracy and sensitivity of the assay for the product of the reporter gene is significantly increased.

(c) The detection system can be made to have high specificity which can be as high as that of a direct measure of the mRNA corresponding to the reporter gene.

Many genes encoding thermostable enzymes are already known, especially those present in extremophile achae-bacteria. The thermostable enzymes have been well characterized (5,6,7,8) and the corresponding genes coding for them are available (9). The gene (lacS) encoding a β-galactosidase (SsB-Gal) from the archeabacterium Sulfolobus solfataricus is preferably used in the present invention. (This microorganism, strain MT4, is available from, for

example, ATCC and DSM). Th enzyme produced by this gene has been purified to homogeneity and extensively characterized (8). A gene library of the microorganism has been constructed in lambda gt11 and the β-galactosidase gene isolated, cloned (9) and expressed in E. coli (10).

The potency of lacS as a reporter gene in eukaryotic systems has been demonstrated in cultured mammalian cells. For this purpose, four different eukaryotic expression vectors, suitable for transient or stable transfection have been constructed. All four vectors contain strong enhancer/promoter sequences which allow high expression of the cloned gene. Figures 1 and 2 are plasmid maps of the four vectors which have been made. The first two plasmids suitable for transient transfection (see Figure 1) were designed as follows.

pSV$_2$-lacS

This plasmid was constructed by substituting the Hind III/Pvu II fragment of the CAT gene in pSV$_2$-CAT(1) using a Hind III fragment containing all the coding sequence of the lacS gene.

pRSV-lacS

The Bam HI region (containing the lacS gene and the SV40 polyadenylation site and intron) from pSV$_2$-lacS was inserted in the Hind III/Bam HI digested pRSV-neo(1), replacing the neo coding sequence by the sequence containing the lacS gene.

Two more plasmids w re constructed for the selection of stable transfectants. These are shown in Figure 2. Both the plasmids possess the neo gene under the control of the SV40 enhancer/promoter system. pRC/RSV-lacS and pRC/CMV-lacS were constructed by inserting the Hind III fragment of LacS at the Hind III site of the polylinkers of, respectively, pRC/RSV and pRC/CMV vectors obtained from INVITROGEN.

Simian CV1 and mouse fibroblast NIH3T3 cell lines were used as host systems. They were transfected by the calcium/phosphate coprecipitation technique. For transient invention, cells were harvested 48 hours after DNA addition and cell lysates were prepared and assayed for enzymatic activity.

For stable transfection CV1 cells transfected with pRC/CMV-lacS were selected by growth in the presence of selection marker G418. To assay the expressed Ss β-gal activity, cell extracts were preincubated at 70°C for ten minutes in the presence of 0.1% SDS to inactivate any endogenous enzymatic activity of the transfected cells. The assay was then performed by the method of Maniatis et al (11) but at 75°C rather than 37°C.

The results of the transient transfection experiments and the expression of the thermostable enzymes in the two cell lines are shown in Figure 3. Non-transfected NIH3T3 and CV1 cells were used as negative

controls giving background activity valu s. Ss β-gal activity was reported in relation to cell number and normalised with respect to CAT activity determined in cells which were cotransfected with the pSV_2CAT plasmid. NIH3T3 cells were transfected with 2μg of pSV_2-CAT or alternatively with 2 or 20μg of pRSV-lacS or pRC/RSV-lacS. CV1 cells were cotransfected with the same amount of the reference plasmid or with 2 or 20μg of pSV_2-lacS or pRC/CMV.

The expression system of the present invention is conveniently provided in the form of a plasmid or other vector comprising the said system, preferably with a polylinker sequence upstream of the reporter gene to facilitate cloning of a desired promoter region to be tested. As described above such plasmids may be used in transient or stable transfection. For control purposes, plasmids or other vectors may be provided having an expression system in which the same reporter gene is under the control of a promoter sequence of known potency.

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CLAIMS

1. A recombinant protein or peptide expression system comprising, as reporter gene, a gene coding for an enzyme or other protein which is stable under conditions in which at least some of the other protein or peptide products of the said system are inactivated.

2. An expression system according to claim 1 in which the said gene codes for an enzyme which is stable under conditions of temperature in which substantially all of the other protein or peptide products of the said system are denatured.

3. An expression system according to claim 2 in which the said gene codes for a thermostable β-galactosidase of Sulfolobus solfataricus.

4. An expression system according to claim 3 in which the said gene is the lacS gene.

5. An expression system according to any one of claims 1 to 4 which also includes a promoter and/or other DNA sequence which does not encode a protein or peptide product but assists in the expression of the reporter gene.

6. A plasmid or other vector comprising an expression system as claimed in any of claims 1 to 5.

7. A plasmid or other vector according to claim 6 having a polylinker sequence upstream of the reporter gene.

Fig.1.

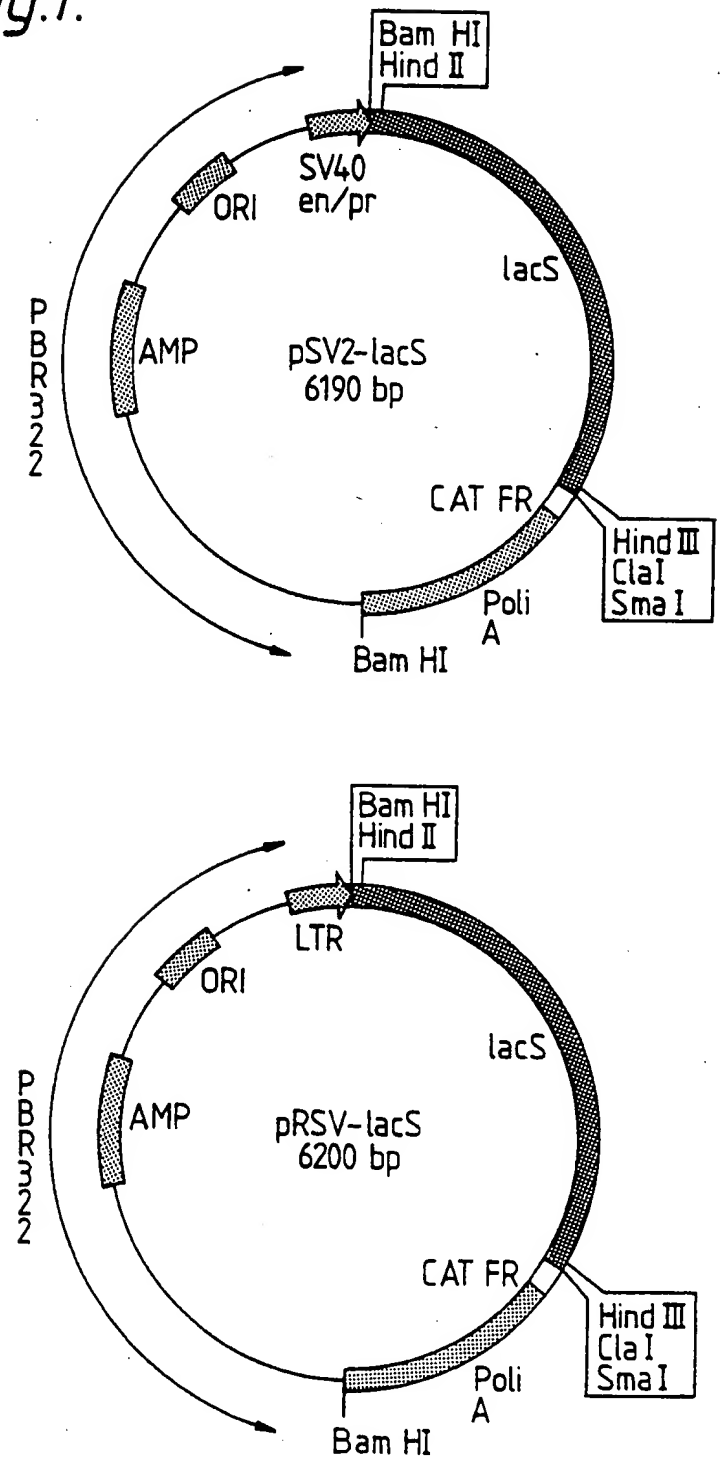
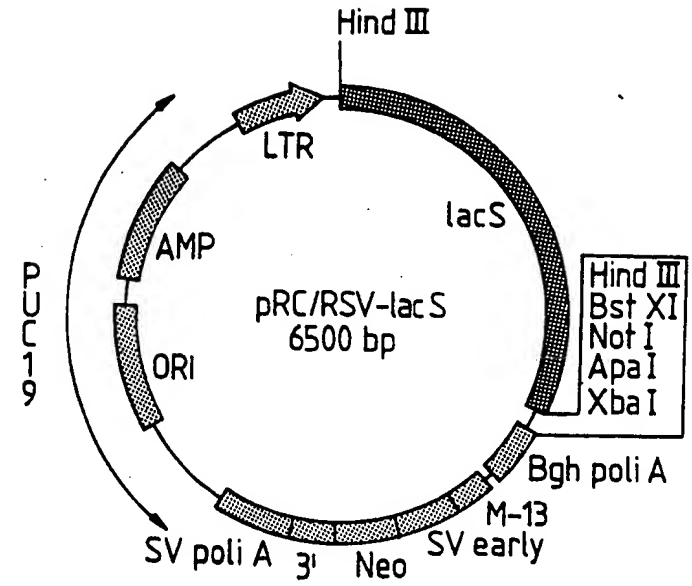
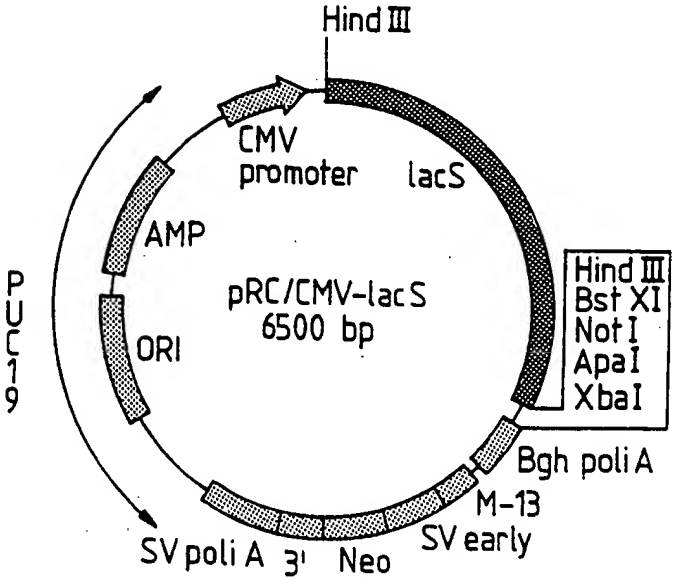

Bam HI
Hind II
SV40
en/pr
ORI
lacS
PBR322
AMP
pSV2-lacS
6190 bp
CAT FR
Hind III
ClaI
Sma I
Poli
A
Bam HI
Bam HI
Hind II
LTR
ORI
lacS
PBR322
AMP
pRSV-lacS
6200 bp
CAT FR
Hind III
Cla I
Sma I
Poli
A
Bam HI

Fig. 2.


Hind III
LTR
lacS
AMP
pRC/RSV-lac S
6500 bp
ORI
PUC 19
Hind III
Bst XI
Not I
Apa I
Xba I
Bgh poli A
M-13
SV early
SV poli A
3'
Neo


Hind III
CMV
promoter
lacS
AMP
pRC/CMV-lac S
6500 bp
ORI
PUC 19
Hind III
Bst XI
Not I
Apa I
Xba I
Bgh poli A
M-13
SV early
SV poli A
3'
Neo

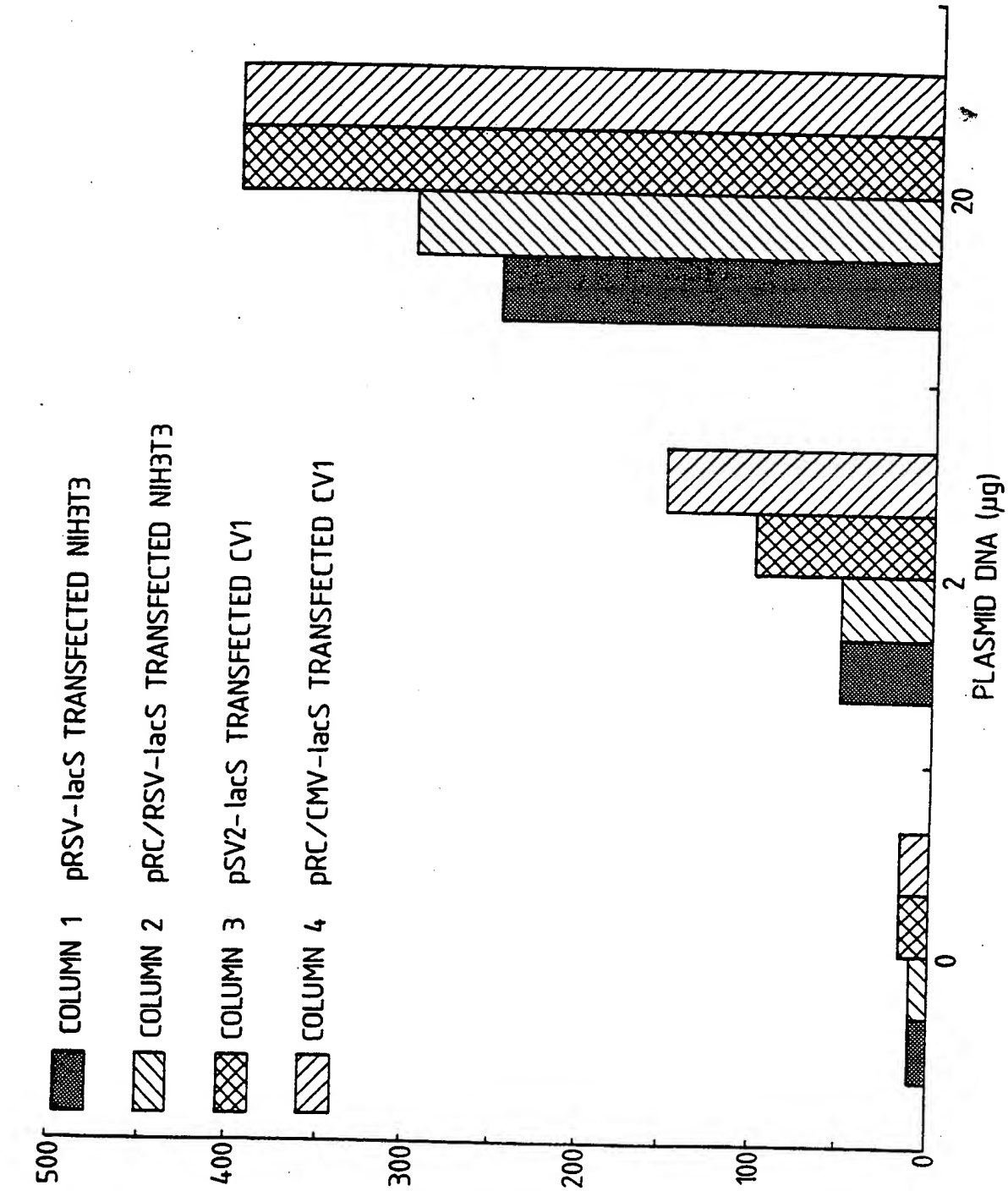

COLUMN 1 pRSV-lacS TRANSFECTED NIH3T3
COLUMN 2 pRC/RSV-lacS TRANSFECTED NIH3T3
COLUMN 3 pSV2-lacS TRANSFECTED CV1
COLUMN 4 pRC/CMV-lacS TRANSFECTED CV1
RELATIVE Ss β-Gal ACTIVITY
500
400
300
200
100
0
0
2
20
PLASMID DNA (µg)

Fig. 3.

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/56; C12N15/67; C12N9/38

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	NATO ASI SERIES A: LIFE SCIENCES vol. 183, 1989, SPRINGER VERLAG, BERLIN, BRD; pages 325 - 331 M.ROSSI ET AL. 'Cloning, sequencing and expression of a new beta-galactosidase from the extreme thermophilic sulfolobus solfataricus' cited in the application --- -/--	1-7

° Special categories of cited documents :[10]

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
15 JANUARY 1993	04.02.93
International Searching Authority	**Signature of Authorized Officer**
EUR PEAN PATENT OFFICE	HORNIG H.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	GENE vol. 106, no. 1, 30 September 1991, ELSEVIER SCI. PUBL., AMSTERDAM, NL; pages 13 - 19 G. BURCHHARDT AND H. BAHL 'Cloning and analysis of the beta-galactosidase-encoding gene from Clostridium thermosulfurogenes EM1' see page 13, line 14 - line 15 see page 18, left column, line 18 - line 21	1-2
Y		3-7
X	CHEMICAL ABSTRACTS, vol. 114, no. 21, 27 May 1991, Columbus, Ohio, US; abstract no. 205470x, M. MORACCI ET AL. 'Cloning and expression as a tool to easily purify a new highly thermostable archebacterial beta-galactosidase' page 666 ;column R ; see abstract	1-4
Y	& SEP. BIOTECHNOL. 2, (PAP. INT. SYMP.), 2ND, 1990, ELSEVIER, LONDON, UK; pages 577 - 582 EDITED BY D.L. PYLE abstract	5-7
Y	NUCLEIC ACID RESEARCH vol. 17, no. 19, 11 October 1989, EYNSHAM, OXFORD, GB; page 7980 S. LITTLE ET AL. 'Nucleotide sequence of a thermostable beta-galactosidase from Sulfolobus solfatarius' the whole document	3-7
Y	GENE vol. 94, no. 2, 15 October 1990, ELSEVIER SCI. PUBL., AMSTERDAM, NL; pages 89 - 94 M.V. CUBELLIS ET AL. 'Isolation and sequencing of a new beta-galactosidase-encoding archebacterial gene' see page 92, left column, line 25 - line 28	3-7

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	MOLECULAR CLONING, A LABORATORY MANUAL 1989, CSH LABORATORY PRESS,LONG ISLAND,US; pages 16.56 - 16.59 SAMBROOK, FRITSCH, MANIATIS 'Srategies for studying gene regulation' cited in the application see page 16.57, line 15 - line 21 ---	3-7
P,X	J. BACTERIOL. vol. 174, no. 3, February 1992, AM. SOC. MICROBIOL.,BALTIMORE, US; pages 873 - 882 M. MORACCI ET AL. 'Expression of the thermostable beta-galactosidase gene from the archaebacterium Sulfolobus solfataricus in Saccharomyces cerevisae and characterization of a new inducible promoter for heterologous expression' see page 874, left column, line 1 - right column, line 46; figures 1,5 -----	1-7